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Mechanical unloading impairs keratinocyte migration and angiogenesis during cutaneous wound healing

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Radek KA, Baer LA, Eckhardt J, DiPietro LA, Wade CE. Mechanical unloading impairs keratinocyte migration and angiogenesis during cutaneous wound healing. *J Appl Physiol* 104: 1295–1303, 2008. First published February 21, 2008; doi:10.1152/japplphysiol.00977.2007.— Although initially thought to improve an individual's ability to heal, mechanical unloading promoted by extended periods of bed rest has emerged as a contributing factor to delayed or aberrant tissue repair. Using a rat hindlimb unloading (HLU) model of hypogravity, we mimicked some aspects of physical inactivity by removing weight-bearing loads from the hindlimbs and producing a systemic cephalic fluid shift. This model simulates bed rest in that the animal undergoes physiological adaptations, resulting in a reduction in exercise capability, increased frequency of orthostatic intolerance, and a reduction in plasma volume. To investigate whether changes associated with prior prolonged bed rest correlate with impaired cutaneous wound healing, we examined wound closure, angiogenesis, and collagen content in *day 2* to *day 21* wounds from rats exposed to HLU 2 wk before excisional wounding. Wound closure was delayed in *day 2* wounds from HLU rats compared with ambulatory controls. Although the levels of proangiogenic growth factors, fibroblast growth factor-2 (FGF-2), and vascular endothelial growth factor (VEGF) were similar between the two groups, wound vascularity was significantly reduced in *day 7* wounds from HLU animals. To further examine this disparity, total collagen content was assessed but found to be similar between the two groups. Taken together, these results suggest that keratinocyte and endothelial cell function may be impaired during the wound healing process under periods of prolonged inactivity or bed rest.

tissue repair; rat; skin

CHRONIC OR NONHEALING WOUNDS have become a significant financial burden on healthcare resources and represent an increasing problem among individuals subjected to extended periods of bed rest. Both Earth-based mechanical unloading and spaceflight studies have uncovered dramatic physiological changes that occur when subjects are exposed to prolonged bed rest or a microgravity environment. Individuals exposed to mechanical unloading, through periods of hypogravity during spaceflight or prolonged bed rest, acclimate to their new physiological state by altering cardiovascular and metabolic systems. Mechanical unloading is associated with reduced plasma volume, cardiac and vascular adaptations, a reduction in exercise capacity, and increased frequency of orthostatic intolerance (25, 41). Various aspects of overall tissue repair have also been assessed following exposure to absolute mechanical unloading conditions during spaceflight and have revealed significant attenuation in the healing of soft tissue, muscle, and bone (8, 19, 30, 49).

Hindlimb unloading (HLU) represents an established animal model for tissue disuse as a means to simulate bed rest through mechanical unloading (39). Mechanical unloading contributes to substantial alterations in the musculoskeletal system, including muscle atrophy, reduced muscle size and strength, and disuse osteoporosis (44, 56). Ligament healing is significantly impaired when the mechanical load is removed following injury, as rats subjected to 7 wk of HLU revealed an impairment of temporal collagen gene expression ligament remodeling compared with ambulatory controls (36), ultimately leading to abnormal mechanical properties and connective tissue morphology. Immunological studies have identified impaired lymphocyte responsiveness and phagocytic capability of neutrophils when normal individuals were subjected to prolonged bed rest (26, 41, 51). It has also been reported that lymphatic function is impaired in rats subjected to mechanical unloading (28). Incidentally, human cutaneous wound healing abnormalities are often observed in diabetic, obese, and aged individuals who experience restricted physical activity (13).

Wound repair involves a complex interaction between cytokines, growth factors, cells, and the extracellular matrix (ECM) throughout three overlapping phases (13, 60). The inflammatory phase represents the initial phase and is distinguished by the deposition of the fibrin clot, platelet degranulation, the infiltration of immune cells, and the robust release of cytokines and growth factors. The subsequent phase, the proliferative phase, entails wound closure, angiogenesis, and matrix deposition, initiated in response to stimulatory factors produced during the inflammatory phase. The remodeling phase is the final phase of wound healing and includes the generation of degradative enzymes, additional matrix proteins, and cross-linking of collagen, processes which can continue for years to months following the initial injury and result in scar formation (5, 13, 52, 60). Ultimately, ~80% of the original integrity of the skin is regenerated on completion of tissue repair during normal physiological wound healing (5). In an uncompromised individual, the wound healing process generally proceeds without complications. However, numerous factors such as age, nutritional status, physical activity, and systemic disease have been associated with abnormal tissue repair (3, 4, 18, 27, 40, 43, 47, 55, 57).

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Recent evidence suggests that weight-bearing physical activity improves the body's ability to utilize nutrients, improves circulation, and promotes the healing of injured tissue (11, 24, 34). Despite this evidence, there has yet to be a study conducted in which the subject is exposed to mechanical unloading before cutaneous wounding to determine the specific effects of mechanical unloading on the wound healing process. Observations from prior studies suggest alterations in healing with concurrent tissue injury and mechanical unloading. However, the increasing frequency of wound incidence in geriatric individuals and/or patients subjected to previous mechanical unloading emphasizes the necessity for a direct investigation into the effects of prior mechanical unloading on cutaneous wound healing in vivo.

The purpose of this study was to investigate the global alterations in cutaneous wound healing induced following previous mechanical unloading. We hypothesized that animals subjected to mechanical unloading before cutaneous wounding would exhibit an overall delay in wound repair. For these studies, we subjected rats to the HLU model 14 days before excisional wounding. Wounds were then analyzed for wound closure, angiogenesis, and collagen content from both hindlimb-suspended and ambulatory control animals. Using this model, we were able to detect significant delays in wound reepithelialization and revascularization, despite normal levels of proangiogenic cytokines and collagen matrix. The data acquired from these studies emphasize the need for novel treatment strategies to optimize wound healing in individuals subjected to prolonged bed rest before cutaneous injury.

MATERIALS AND METHODS

Animal subjects. Animal experimentation was conducted in accordance with the guidelines of the National Aeronautics and Space Administration (NASA) Institutional Animal Care and Use Committee. All experiments were reviewed and approved by the NASA/Ames Research Center Institutional Animal Care and Use Committee and conducted in compliance with the National Research Council's *Guidelines for the Care and Use of Laboratory Animals.*

Hindlimb unloading. Mature male Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing between 350 and 375 g were transported to Ames Research Center. On arrival, rats were housed in standard vivarium cages (47 × 26 × 21 cm) lined with corncob bedding and maintained under the following conditions: light cycle, 12 h, light beginning at 0600, and 12 h dark, 23 ± 1°C, and 30–50% humidity. Food and water was available ad libitum. Animals were weighed daily after arrival to monitor body weight for group assignments. The rats were randomly assigned to a single nonselected group (unmanipulated), or either HLU or control ambulatory groups at one of six time points: baseline and 3, 7, 10, 14, and 21 days postwounding. All groups had six rats within each treatment group. HLU animals and their controls were transferred to specialized suspension cages for unloading 3 days before HLU to acclimate to the suspension cages. Rats in the HLU group were then subjected to HLU for 14 days before wounding to allow for physiological acclimation to mechanical unloading. For the HLU model, animals had a castlike apparatus applied to their tail. To allow for free movement about the cage, the cast was attached to a swivel anchored to the top of the cage, permitting 360° range of motion. Animals were suspended in a 30° head down-tilt position (39). Animals were weighed daily to monitor changes in body mass for the duration of the study. For the wounding procedure, all animals used in the study were anesthetized with isoflurane (2.5% in O_2). When completely anesthetized, each animal had its dorsum shaved and six full-thickness excisional punch wounds were placed using a 3-mm biopsy punch (Acu Punch, Acuderm, Lauderdale, FL). Animals were returned to the HLU model immediately after recovery from the wounding procedure and remained unloaded until they were killed. At specific time points after injury, animals were killed, and individual wounds were harvested from the pelt using a 5-mm punch biopsy instrument and snap-frozen in Optimal Cutting Temperature (OCT) compound (Sakura Finetechnical, Tokyo, Japan). For the hydroxyproline, FGF-2, and VEGF analysis, the level was normalized to wet weight by dividing by the average wet weight (mg) as determined for each time point. For each of the different excisional wound analyses, one of the six wounds was randomly selected from each animal, and this single wound was utilized for that unique analysis. Unwounded skin from the nonselected group was also included for baseline levels. Analgesics (buprenorphine, 0.03 ml) were administered subcutaneously immediately following wounding. Antibiotics were not required, as postoperative infection is exceptionally rare in this model system. All animals were killed by decapitation while under anesthesia (isoflurane, 2.5% in O_2). Skin samples were immediately excised and frozen in OCT medium or snap-frozen in liquid nitrogen. Whole blood was collected via heart puncture at the time of dissection while rats were under anesthesia to determine any changes in blood volume. The right and left soleus, gastrocnemius, and tibialis anterior muscle groups were also harvested to confirm the efficacy of the unloading model and identify muscular atrophy as an indication of mechanical unloading.

Analysis of wound reepithelialization. Wound reepithelialization of excisional wounds ($n = 6$ per group, 1 wound per rat) was measured using histomorphometric analysis of 10-μm tissue sections from the middle of the wound as described previously (48). Briefly, sections were stained with hematoxylin and eosin. The percentage of reepithelialization [(distance covered by epithelium/distance between wound edges) × 100] was calculated for each section and performed blinded. The average reepithelialization between two wound sections for each animal was used as a unique value. Instead of using a separate group, we generated *day 2* wounds by administering two of six wounds 1 day after the initial four wounds were made on rats assigned to the *day 3* wound group, since reepithelialization analysis was the only analysis that required a *day 2* time point.

Analysis of wound collagen content by hydroxyproline quantification. Hydroxyproline is a major component of the protein collagen and, thus, is used as an indicator to determine collagen levels in tissue. The hydroxyproline content of single individual wounds ($n = 6$ per group, 1 wound per animal) was determined according to a standard protocol (59). Each wound was harvested from the pelt using a uniform 5-mm biopsy punch. Because the specimens do contain some adjacent normal skin, this analysis compares relative differences between treatment groups rather than absolute values. Since the amount of normal skin is nearly identical in all samples, any observed differences were expected to reflect that of the wound and not normal skin. All reagents for this assay were purchased from Sigma Chemical (St. Louis, MO). Briefly, frozen tissue was hydrolyzed in 2 ml of 6 N HCl overnight at 110°C. The reaction was neutralized with 2.5 N NaOH and diluted 40-fold with MilliQ water. One milliliter of a 0.05 M chloramine T solution was added to 2 ml of the neutralized/diluted solution and incubated for 20 min at room temperature. One milliliter of 3.15 M perchloric acid was added, and the solution was incubated for 5 min at room temperature. One milliliter of 20% *p*-dimethylaminobenzaldehyde was subsequently added, and the mixture was incubated for 20 min at room temperature. The samples were then cooled with cold tap water. The amount of hydroxyproline was determined by comparison to a standard curve measured spectrophotometrically at an absorbance of 557 nm. Samples were analyzed in duplicate.

Analysis of wound angiogenesis. Wound vessel density ($n = 6$ per group, 1 wound per animal) was determined following immunohistochemical staining with an antibody specific to platelet-derived endothelial cell adhesion molecule-1 (PECAM-1or CD-31) (PharMingen, San Diego, CA), a marker for vascular endothelial cells, as previously

described (45). Briefly, 10-μm sections were fixed in acetone for 15 min at room temperature, pretreated with 3% hydrogen peroxide in methanol to block endogenous peroxidase activity, and then blocked for nonspecific binding with 50 μl normal mouse serum (1:100; Sigma). Secondary antibody (biotinylated mouse-α-rat IgG, Jackson, West Grove, PA) incubations were performed for 30 min each. Sections were subsequently incubated for 30 min with avidin-biotin horseradish peroxidase complexes (ABC Vectastain, Vector Laboratories, Burlingame, CA). Color development was performed with 3,3′-diaminobenzidine (Kirkegard and Perry, Gaithersburg, MD), and sections were counterstained with Harris hematoxylin. Image Analysis software was utilized to determine vessel staining (Scion Image Software; Optronics Engineering Software). The PECAM-positive area within the wound bed was assessed, and percent vascularity was calculated as (PECAM-positive area/total wound bed area) × 100%.

The average percent vascularity (vessel density) between two wound sections for each animal was used as a unique value.

Analysis of wound FGF-2 and VEGF levels. Dermal FGF-2 and VEGF levels were determined using a human FGF-2 ELISA kit or murine VEGF164 (mVEGF164) ELISA kit (Quantikine R&D Systems, Minneapolis, MN) as previously described (53). The amount of growth factor in unique individual wounds (1 from each animal) was determined and normalized to the average wet weight of wounds for each specific time point. Because both FGF-2 and VEGF are nearly undetectable in normal skin, any adjacent normal tissue that was included in the sample added only negligible amounts to the total measured value. To perform this analysis, individual wounds ($n = 6$ per group, 1 wound per animal) were removed from the pelt with a 5-mm biopsy punch and were placed in 1 ml of homogenization buffer [1 protease inhibitor tablet (Boehringer-Manheim) in 50 ml D-PBS (Gibco-BRL)]. Wounds were homogenized at high speed for 10–15 s. The homogenate was then sonicated at 30% power for 10 s. Samples were then centrifuged at 800 *g* for 2 min at 4°C, transferred to 1.5-ml centrifuge tubes, and centrifuged at 2,000 *g* for 10 min at 4°C. The supernatant was then filtered through a 1.2-μm-pore syringe filter and snap-frozen in liquid nitrogen. Samples were analyzed in duplicate. Both ELISA assays were performed according to the manufacturer's protocol. Wound VEGF and FGF-2 levels were normalized to total wet weight of the wound and derived from a standard curve read at an absorbance of 450 nm in a spectrophotometer.

Histopathological analyses. Wounds from control or HLU rats were collected using a punch biopsy and embedded in OCT. At a later date, the wounds were defrosted and fixed in 10% neutral buffered formalin solution for 48 h. Formalin-fixed tissues were processed routinely, embedded in paraffin, sectioned at ~5 μm, stained with hematoxylin and eosin stain, and evaluated for microscopic lesions by a board-certified veterinary pathologist. The histological sections were reviewed and scored in a blinded fashion with standard light microscopy on an Olympus BX41 microscope. All layers of the skin from epithelium to panniculus were evaluated on a 0–4 scale for both degree of healing and amount and type of inflammation.

The following parameters were analyzed in the epidermis, superficial dermis, dermis, and panniculus carnosus by arbitrary units in a blinded fashion: *1*) infiltration of fibrin, neutrophils, macrophages, lymphocytes, and plasma cells: 1 = minimal, 2 = mild, 3 = moderate, 4 = severe; and *2*) fibroblast, fibrocyte, and myofibroblast populations, and collagen organization: 1 = mature fibrocytes, organized collagen; 2 = fibrocytes, moderately organized collagen; 3 = fibroblasts, minimally organized collagen; 4 = immature fibroblasts, minimal collagen formation.

Statistical analysis. Data were analyzed using GraphPad Prism, version 2.1 (GraphPad Software, San Diego, CA). The means and SE were calculated for each data set. Data sets were analyzed by two-way ANOVA and a Bonferroni post test. P values <0.05 were considered significant.

RESULTS

Body mass during experimental period. Male rats initially weighing between 350 and 375 g were randomly assigned to either control or HLU groups (30 rats per group) and then further subdivided into five wounding groups (6 rats per group) as described. A timeline of unloading events and tissue collection is depicted in Fig. 1. Initial body mass of the rats (baseline) did not differ between assigned groups in standard vivarium cages (Fig. 2). Rats were then placed in unloading cages 3 days before unloading or control conditions to acclimate to the specialized cage, during which body mass gain was similar between groups. After 3 days in unloading cages, rats were subjected to HLU or remained ambulatory. During the 14-day unloading and up to 3 days postwounding, the body mass of the ambulatory control rats was consistently higher than that of the HLU group. However, no significant reduction in body mass was observed in the HLU group between *day 7* after unloading and time of dissection (data not shown). No significant changes in blood volume were identified between groups (data not

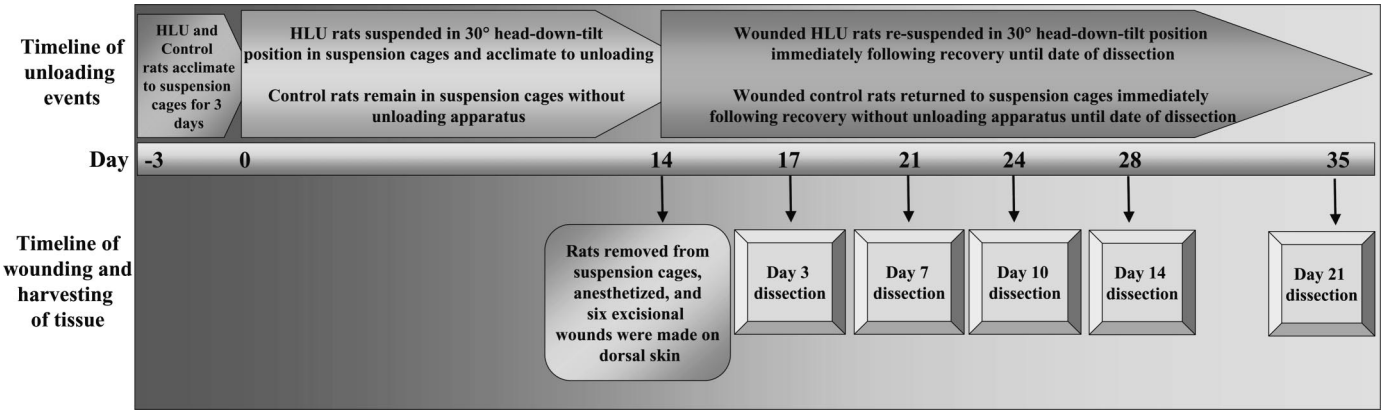

Fig. 1. Timeline of unloading, wounding, and harvesting of tissue. Rats selected for the hindlimb unloading (HLU) or control groups were placed in suspension cages and allowed to acclimate to the cages for 3 days (*day −3* to *day 0*). On *day 0*, HLU rats were subjected to unloading using a tail suspension apparatus, while control rats remained in suspension cages. HLU rats were allowed to acclimate to the unloading for 14 days (*days 0–14*). HLU rats were removed from the unloading apparatus on *day 14* for wounding but were resuspended immediately after recovery and remained suspended for the duration of the study. Rats were wounded at *day 14* after acclimation to unloading. *Day 3, day 7, day 10, day 14,* and *day 21* wounds, blood, and right and left soleus, gastrocnemius, and tibialis anterior muscles were collected during dissection at the indicated time points.

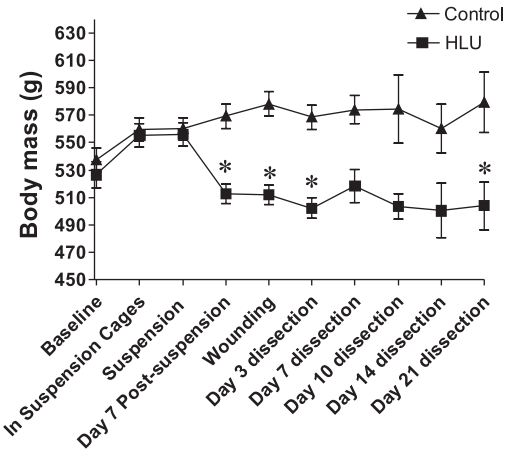

Fig. 2. Effect of hindlimb unloading on body mass at specific points during the experimental period. Control, ambulatory rats; HLU, suspended rats. Values represent the mean body mass (g) ± SE; $n = 30$ per group. *$P < 0.001$ vs. control by 2-way ANOVA and a Bonferroni posttest.

shown). However, at each time point the weight of the soleus and gastrocnemius muscles from HLU rats was significantly less than that of control rats, confirming the efficacy of the HLU model indicated by hindlimb muscle atrophy (data not shown) (39).

Hindlimb unloading delays wound closure. To determine if simulated bed rest influenced wound reepithelialization, we examined epithelial migration across the wound bed. At *days 2* and *3* postwounding, wound sections obtained from hindlimb exposed rats (Fig. 3*B*) were significantly less reepithelialized compared with those of ambulatory controls (Fig. 3*A*). Arrows indicate the distance migrated by the keratinocytes across the wound bed. Normal skin is seen on either side of the wound, indicated by dark pink staining. However, by *day 7* there was no significant difference in wound coverage between the two treatment groups, with wounds from both treatment groups completely reepithelialized by *day 7* (Fig. 3*C*).

Hindlimb unloading inhibits wound angiogenesis. Since we observed a delay in wound closure by keratinocytes, we postulated that this may also prompt alterations in endothelial cell function. Thus we next sought to determine if mechanical unloading would impair wound angiogenesis. Photographs of tissue sections of wounds from hindlimb exposed rats exhibited fewer vessels compared with wounds from ambulatory controls. Vasculature from wounds of control rats appears to migrate toward the epithelium (Fig. 4*A*). In contrast, vasculature from wounds of HLU rats is more punctate and lacks directional migration toward the epithelium (Fig. 4*B*). Analysis of wound vascularity (Fig. 4*C*) revealed significantly lower wound vascularity at *day 7* postwounding; however, no significant difference was observed at *days 10, 14,* or *21* postwounding. Although the percent vascularity was reduced in wounds from HLU rats, the pattern of angiogenesis was normal, with peak vascularity at *day 7* and vascularity equivalent to uninjured skin by *day 21*.

Hindlimb unloading does not induce changes in proangiogenic cytokines during wound healing. To further assess the effect of hindlimb unloading on reduced wound angiogenesis, we examined the levels of two key proangiogenic cytokines in wounds. To ensure that any alterations in the levels of FGF-2 and VEGF would not be neglected, we chose to assess the levels of these cytokines at time points directly before and after their normal peak to include *days 3, 7*, and *10* postwounding (22, 45). No significant differences in the levels of FGF-2 (Fig. 5*A*) or VEGF (Fig. 5*B*) were observed between control or HLU rats.

Hindlimb unloading does not affect wound hydroxyproline content. After observing a significant reduction in wound vascularity, despite similar levels of proangiogenic cytokines, we next determined if this may have been due to a reduction in wound collagen levels. To determine the level of collagen content in the wound, we quantified the total levels of collagen in wounds from ambulatory or HLU-treated rats using hydroxyproline as an index for total collagen content (Fig. 6). Hydroxyproline content was not significantly altered between either group at any time point following wounding. The pattern of hydroxyproline content, indicative of the amount of collagen being deposited in the wound bed, follows that of a normal healing wound, with levels of hydroxyproline significantly below that of normal, uninjured skin in *day 3* wounds and

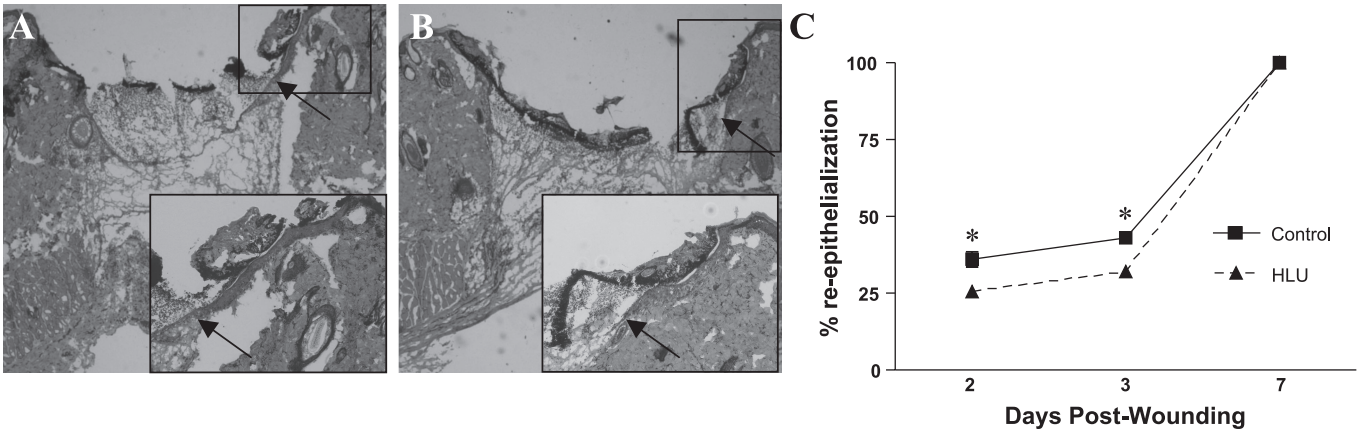

Fig. 3. Hindlimb unloading delays wound reepithelialization. *Day 2* wounds from control rats (*A*) or HLU rats (*B*) were photographed at ×10 magnification. *Inset*: boxed area at ×40. Arrow indicates the distance migrated by keratinocytes. *C*: percentage of reepithelialization [(distance covered by epithelium/distance between wound edges) × 100] was calculated at *days 2, 3,* and *7* postwounding. Reepithelialization of wounds from hindlimb-suspended rats (dashed line) was significantly delayed at *days 2* and *3* compared with wounds from ambulatory controls (solid line). Data are expressed as mean percentage reepithelialization ± SE; $n = 6$ per group. *$P < 0.05$ by a 2-way ANOVA and Bonferroni post test.

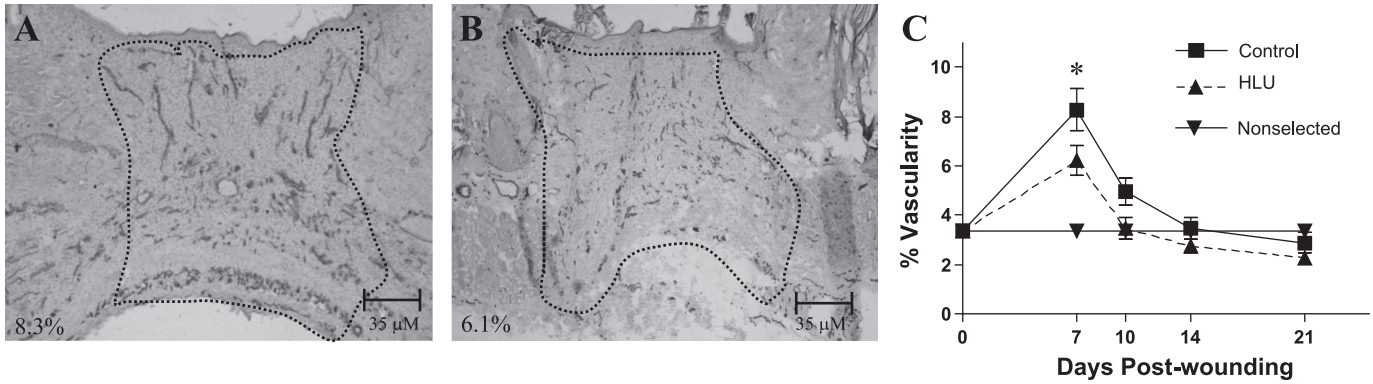

Fig. 4. Hindlimb unloading reduces wound vascularity. Histology of *day 7* wound sections from ambulatory controls (*A*) and HLU rats (*B*). Frozen sections from *day 7* to *day 21* were stained with anti-PECAM-1 antibody. The area of the wound bed that was used for vascular density analysis is indicated by the dotted line. The percent vascularity for each section is depicted in the bottom left corner. *C*: percent vascularity (vessel density) was calculated for wounds from HLU (▲) or ambulatory controls (■) using image analysis software (Scion Image). Nonselected, normal skin (▼). Data are expressed as mean percent vascularity ± SE; $n = 6$ per group. *$P < 0.05$ for *day 7* by 2-way ANOVA and a Bonferroni posttest.

increases above normal, uninjured skin by *day 7* in both controls and HLU rats (45). In addition, total hydroxyproline content in uninjured skin was also not affected by HLU (data not shown).

Differences in wound pathologies induced by HLU. Wounds from *days 3, 7, 10,* and *14* rats were subjected to histological analysis to detect differences in inflammatory and dermal pathology (Table 1). Wounds from HLU rats had greater neutrophil infiltration within the epidermis and dermis compared with control rats. Furthermore, wounds from HLU rats exhibited a greater population of fibrocytes, or inactive fibroblasts, compared with fibroblasts. However, this did not result in a decrease in total wound collagen content in our studies (Fig. 6), as wounds from HLU rats also exhibited moderate collagen organization within the dermis. Finally, wounds from HLU rats exhibited only mild to minimal lymphocyte and plasma cell infiltrate compared with control rats.

DISCUSSION

The results of this study demonstrate for the first time that previous mechanical unloading promotes global alterations in cutaneous wound healing. Both mechanical loading and physical activity denote bodily movement by skeletal muscles that results in physiological energy expenditure. Although the current belief is that one must undergo vigorous exercise to achieve health benefits, moderate-intensity activities such as walking and resistance training have been shown to considerably improve overall health and healing capability. The recumbent position as part of prolonged bed rest contributes to a reduction in muscular force on bone, energy utilization, and hydrostatic pressure (25). Previous studies have indicated that mechanical unloading dramatically increases the risk for developing secondary complications, including muscle degeneration, cardiovascular dysfunction, thrombosis, diabetes, and chronic wounds (2, 6, 7, 17, 32). At the cellular level, mechanical unloading has been shown to influence gene expression, signal transduction pathways, and proliferative capacity (9, 14, 15, 38). Both exogenous as well as physiological factors contribute to specific cellular alterations associated with abnormal wound healing (37, 53). As additional factors emerge as potential mediators of aberrant tissue repair, the obligation to develop novel interventions designed to improve healing

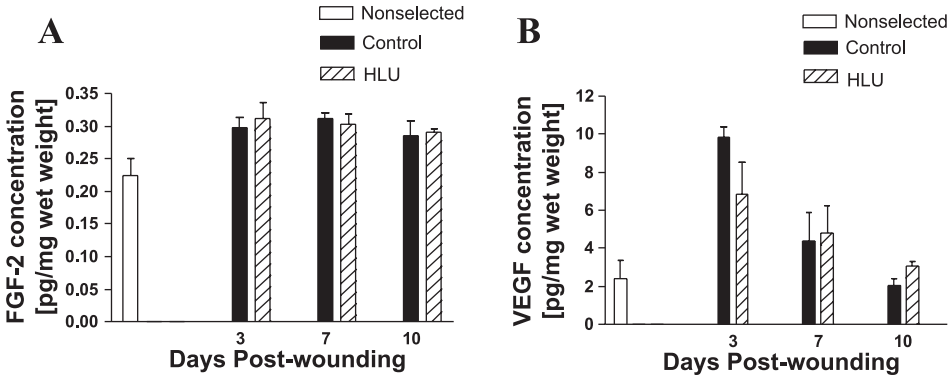

Fig. 5. Hindlimb unloading does not alter the levels of proangiogenic cytokines in wounds. *A*: wounds from *days 3, 7,* and *10* postwounding were collected and homogenized. FGF-2 levels in wound homogenates from hindlimb unloaded (hatched bars) and ambulatory controls (black bars) was determined by ELISA. The peak of FGF-2 expression occurred at *day 7* in wounds from both treatment groups. Data are expressed as mean FGF-2 in pg per mg of the average wet weight for each day ± SE; $n = 6$ per group. Nonselected, normal skin (white bars). $P > 0.05$ for all time points by 2-way ANOVA and a Bonferroni posttest. Analysis was performed in duplicate. *B*: wounds from *days 7, 10*, and *14* postwounding were collected and homogenized. VEGF levels in wounds from HLU (hatched bars) and ambulatory controls (black bars) were determined by ELISA. The peak of VEGF expression occurred at *day 7* in wounds from both treatment groups. Data are expressed as mean VEGF in pg per mg of the average wet weight for each day ± SE; $n = 6$ per group. $P > 0.05$ for all time points by 2-way ANOVA and a Bonferroni posttest.

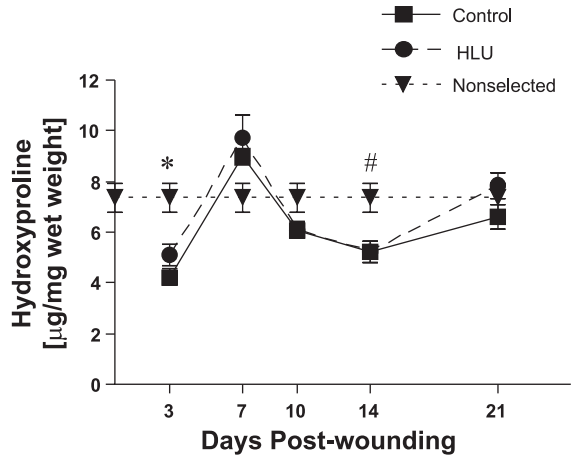

Fig. 6. Hindlimb unloading does not affect wound collagen content. Wounds from HLU and control rats from *day 3* to *day 21* were collected and hydrolyzed. Hydroxyproline content, used as an index for the presence of collagen, was determined in wounds from HLU (dashed line) or ambulatory controls (solid line) using a standard biochemical assay. Data are expressed as mean hydroxyproline content in μg per mg of the average wet weight for each day ± SE; $n = 6$ per group. $P > 0.05$ between control and HLU rats for all time points by 2-way ANOVA and a Bonferroni posttest. $*P < 0.001$, $\#P < 0.05$ vs. nonselected.

becomes progressively more apparent. Despite the clinical evidence that mechanical unloading and prolonged bed rest may be detrimental toward tissue repair, the effects on wound repair using an in vivo model have yet to be fully elucidated. The results from our studies indicate that the normal function of both keratinocytes and endothelial cells is impaired during wound healing in the presence of mechanical unloading.

Nutritional status plays a significant role in the wound healing process, as protein requirements increase to provide the necessary components for protein synthesis. For instance, malnourished patients often present with an increase in wound dehiscence, demonstrating that adequate protein, lipid, and vitamin requirements are met for normal regeneration of the ECM (50). Since we did not observe any significant differences in protein parameters, such as total collagen content or levels of proangiogenic cytokines, and because the decrease in body mass normalized before wounding, we suspect that the nutritional status of the HLU rats did not contribute significantly to the observed impairment of wound healing in these studies and suggests a more direct effect of mechanical unloading on specific cell types.

A fundamental component of normal tissue repair requires the restoration of the epithelial barrier to impede microbial colonization. Although no studies have been conducted in an effort to assess the consequence of mechanical unloading on keratinocyte function in skin, there is evidence suggesting that epithelial migration and proliferation are altered during mechanical unloading using HLU. For instance, corneal epithelial wounds were created in hindlimb suspended and control mice to characterize the effects of mechanical unloading on reepithelialization. However, in these experiments the mice were subjected to control conditions or HLU only 7 days before implantation of corneal sponges, whereas our studies allowed for acclimation to the HLU model for 14 days. Nonetheless, the reepithelialization of corneas in this study was significantly delayed by 6–12 h in hindlimb-suspended mice compared with their ambulatory controls (33). This observation was further assessed, and we found that this impairment of the proliferative phase correlated with a significant reduction in both the rate and magnitude of neutrophil migration into the central regions of the corneal wound. Moreover, the expression of key genes involved in macrophage activity was also significantly reduced in the hindlimb-suspended rats. Since macrophage influx is considered a rate-limiting step during tissue repair, alterations in macrophage influx may have profound effects on various stages of wound healing, particularly the proliferative phase (20, 21, 45). Interestingly, wounds from HLU rats exhibited greater neutrophil infiltration within the epidermis and dermis. Consequently, this may also account for the delay in wound closure, as evidence has suggested that excess inflammation inhibits keratinocyte migration (23). Thus the delay in wound reepithelialization in the present study suggests that the ability of dermal keratinocytes to migrate across the wound bed is impeded by alterations in keratinocyte function. However, these data suggest that the prolonged mechanical unloading delays but does not inhibit keratinocyte migration across the wound bed. Tissue perfusion is critical for both extravasation of immune cells into the wound for host defense, as well as to provide sufficient oxygenation required for fibroblast proliferation and angiogenesis. Potentially, alterations in fluid shifts that occur during mechanical unloading, such as during spaceflight or prolonged bed rest, may modify normal mechanical forces acting on specific cell populations involved in tissue

Table 1. *Differences in wound pathologies observed between control or HLU rats*

Factor	Wound	Control	HLU
Inflammation			
Dermal	*Day 3*	Minimal to mild neutrophil and macrophage infiltrate Moderate to mild lymphocyte and plasma cell infiltrate	Mild to moderate neutrophil infiltrate Minimal to mild lymphocyte and plasma infiltrate
Epidermal	*Day 7*	Mild lymphocyte and plasma cell infiltrate	Mild lymphocyte and plasma cell infiltrate; Severe neutrophil infiltrate
Extracellular matrix			
Panniculus carnosus	*Day 3*	Minimal or immature collagen present; fibroblasts and myoblasts present	Mostly immature myfibroblasts present; minimal collagen formation
Superficial dermis	*Day 7*	Fibroblasts present; minimal collagen organization	Fibrocytes present; moderate collagen organization
Dermis	*Day 7*	Fibroblasts present; minimal collagen organization	Fibrocytes present; moderate collagen organization

Histological assessment of wound pathology in wounds from control or hindlimb-unloaded (HLU) rats. Data represent arbitrary differences in inflammation (epithelial and dermal) or extracellular matrix (fibroblast population and collagen organization). Only differences between the two groups are indicated.

repair. If so, then the advantage of mechanical loading may be more dynamic in that it provides both a fluid and physical component to the regulation of cellular function. During unloading, there is a cephalic fluid shift and exclusion of the head-to-toe hydrostatic pressure gradient that promotes orthostatic intolerance, altered tissue perfusion, reduced aerobic capacity, and decreased peripheral vascular resistance (16, 28, 46, 58). Patients subjected to a 6° head-down tilt for 14 days showed decreased cutaneous vasodilator capacity, which was preserved if the subject participated in daily aerobic exercise training during the study (18). Furthermore, rats subjected to HLU exhibited significant hypotension and bradycardia, enhanced vasoconstriction, and vascular resistance (35, 58), indicating that variations in orthostatic homeostasis have the potential to elicit systemic alterations that can impair the wound-healing process.

During tissue repair, keratinocytes secrete various factors that participate in the signaling of endothelial cells in an effort to promote revascularization of the wound bed. Both FGF-2 and VEGF are potent inducers of both physiological and pathological angiogenesis, with VEGF being the most critical cytokine for wound angiogenesis (10, 22, 29, 45). Although the reduction in VEGF was not statistically significant at *day 3* ($P = 0.12$), there was a trend toward a decrease in wound VEGF levels, which may be sufficient enough to delay the angiogenic signal for initiation of angiogenesis. The reduction of angiogenesis despite near-normal levels of proangiogenic cytokines in the present study suggests that mechanical unloading may also have a direct effect on endothelial cell function. Endothelial cells are highly sensitive to changes in biomechanical homeostasis, as they function as regulators of blood flow, immune cell adhesion, coagulation cascade, and vascular smooth muscle cell growth (1, 42, 54). Because angiogenesis is such a tightly controlled mechanism, minute changes in hemodynamics or mechanical forces may antagonize endothelial cell proliferation, a critical element of physiological angiogenesis during wound healing. Furthermore, recent studies have suggested that mechanical unloading considerably impacts endothelial cell function. Monocyte adhesion to endothelial cells was found to be significantly upregulated following HLU, which may impede normal function of endothelial cells and promote an increase in inflammation within the wound bed (31). Moreover, endothelial cells exposed to mechanical unloading were found to downregulate IL-1α, a potent inhibitor of endothelial cell growth, as well as baseline levels of actin, suggesting an adaptation mechanism to restrict angiogenic capacity (12). Concurrently, the lymphatic dysfunction in HLU rats reported by Gashev et al. (28) may further limit the migrational capacity of lymphocytes to the wound bed, thus diminishing the reservoir of growth factors required for normal physiological angiogenesis during wound repair.

VEGF and FGF-2 are both potent inducers of physiological angiogenesis (22). Despite a normal expression pattern of both VEGF and FGF-2 in HLU rats, wound vascularity was still reduced. Nonetheless, wounds from HLU rats demonstrated fewer lymphocytes and plasma cell infiltrate compared with control rats. Since both lymphocytes and plasma cells have been implicated in promoting angiogenesis, perhaps the reduction in lymphocyte and plasma cell numbers within the wound milieu restricts the production of critical factors other than VEGF or FGF-2 required for angiogenesis. Furthermore, microgravity has been implicated in cell survival through alterations in VEGF receptor-2 (VEGFR2) signaling. VEGFR2 has a lower affinity for VEGF compared with VEGF receptor-1 (VEGFR1), but has a direct involvement in endothelial cell signaling, resulting in endothelial cell mitogenesis required for angiogenesis (29). These data in conjunction with the present study suggest a mechanism by which wound angiogenesis is inhibited during mechanical unloading.

Regeneration of the ECM is essential to restore the integrity and elasticity of the injured dermis. As the wound healing process proceeds, regeneration of the extracellular matrix and angiogenesis occur simultaneously, as endothelial cell migration and proliferation employ the ECM as a provisional matrix on which to migrate (13). Rats implanted with polyvinyl acetal sponge discs and subjected to 10 days of spaceflight exhibited a 62% reduction in collagen content compared with those rats subjected to normal gravity conditions (19). For this analysis, the sponge is designed to act as a model of wound healing, as it represents a defined matrix into which growth factors and cells can infiltrate. In these studies, the sponges were infused with pellets designed to release supraphysiological levels of growth factors, FGF-2 and PDGF. Hence, the observed differences may have been apparent because of a more robust fibroblast response mediated by the presence of excess growth factor. In our studies, only physiological levels of FGF-2 were present in the wound, which may explain why no differences were detected. Furthermore, the rats used in our studies were subjected to mechanical unloading 14 days, thus allowing for systemic physiological changes to occur before wounding. Interestingly, rats subjected to HLU demonstrated impairment in the time course of collagen gene expression events during wound repair remodeling of the medial collateral ligament (36). At 7 wk posthealing, the HLU rats exhibited significantly depressed collagen type I, type III, and type V gene expression levels compared with ambulatory control animals, although the levels of hydroxyproline were similar. This suggests that for our results, differences in the genotypic ratios of dermal collagens may contribute to further defects in wound healing that would not be detected by quantitating total hydroxyproline levels in skin wounds. Potentially, prolonged disuse could promote alterations in fibroblast function with respect to appropriate synthesis and temporal expression of specific collagen subtypes during healing.

Limitations to the study. There are limitations to this study that should be addressed in future investigations. While the HLU rats normalized to their conditions, as assessed by stabilization of body mass, the overall reduction in total body mass compared with control rats may be a minor factor that contributed to the delay in wound closure and angiogenesis. Possibly, we may have missed the peak of VEGF production in our analyses, which may have indicated a statistically significant reduction in VEGF before *day 3*. Furthermore, because the tissue was required to be in a hydrated state for homogenization, there may have been changes in VEGF, FGF-2, or hydroxyproline levels that were not detected due to the normalization of tissues that were different in their initial wet weights.

Future directions. Further studies are required to determine if the delay in keratinocyte migration observed in our studies is indirect due to inflammatory changes within the wound milieu or directly through alterations in keratinocyte signal transduc-

tion. Specifically, it would be of interest to determine whether subpopulations of cells (i.e., macrophages, neutrophils) from HLU animals exhibit a change in localization or phenotype, which may contribute the observed defects in keratinocyte migration and endothelial cell function. Although no significant differences in total collagen content, as measured by hydroxyproline levels, were observed in HLU rats despite a greater amount of immature fibrocytes, further studies are required to determine if differences may exist with respect to wound breaking strength, scarring, the extent of collagen organization, or fibril diameter. In addition, the spatial and temporal expression of collagen subtypes could provide further evidence of perturbations in matrix remodeling during HLU.

Perspectives

The data acquired from these studies underlines the importance of mechanical loading in the maintenance of normal cellular function during tissue repair. However, at the present time, the mechanism behind the observed suppression of normal keratinocyte and endothelial cell function needs to be further investigated. These data highlight the complexity in attempting to elucidate the underlying mechanisms by which mechanical unloading directly influences tissue repair. Ultimately, these results emphasize the benefits of weight-bearing physical activity on the healing process and may shed light on specific pathological changes that occur in patients subjected to extended periods of bed rest.

DISCLAIMER

REFERENCES

1. **Aird WC.** Spatial and temporal dynamics of the endothelium. *J Thromb Haemost* 3: 1392–1406, 2005.
2. **Allen C, Glasziou P, Del Mar C.** Bed rest: a potentially harmful treatment needing more careful evaluation. *Lancet* 354: 1229–1233, 1999.
3. **Ashcroft GS, Horan MA, Ferguson MW.** Aging alters the inflammatory and endothelial cell adhesion molecule profiles during human cutaneous wound healing. *Lab Invest* 78: 47–58, 1998.
4. **Ashcroft GS, Horan MA, Ferguson MW.** Aging is associated with reduced deposition of specific extracellular matrix components, an upregulation of angiogenesis, and an altered inflammatory response in a murine incisional wound healing model. *J Invest Dermatol* 108: 430–437, 1997.
5. **Bernstein E, Bolten LL, Mauviel A, Frank T, McGrath JA, Uitto J.** *Principles of Cutaneous Surgery*. New York: McGraw Hill, 1996.
6. **Blanc S, Normand S, Pachiaudi C, Duvareille M, Gharib C.** Leptin responses to physical inactivity induced by simulated weightlessness. *Am J Physiol Regul Integr Comp Physiol* 279: R891–R898, 2000.
7. **Blanc S, Normand S, Pachiaudi C, Fortrat JO, Laville M, Gharib C.** Fuel homeostasis during physical inactivity induced by bed rest. *J Clin Endocrinol Metab* 85: 2223–2233, 2000.
8. **Bolton L, Zaki G, Monte K, Durham S, Monticello TM, Hudak J, Shannon R, Wilfinger WW, Vasko AJ.** Tissue repair in space. *Wounds: Compend Clin Res Pract* 9: 127–142, 1997.
9. **Boonstra J.** Growth factor-induced signal transduction in adherent mammalian cells is sensitive to gravity. *FASEB J* 13, *Suppl*: S35–S42, 1999.
10. **Brown LF, Yeo KT, Berse B, Yeo TK, Senger DR, Dvorak HF, van de Water L.** Expression of vascular permeability factor (vascular endothelial growth factor) by epidermal keratinocytes during wound healing. *J Exp Med* 176: 1375–1379, 1992.
11. **Buckwalter JA, Grodzinsky AJ.** Loading of healing bone, fibrous tissue, and muscle: implications for orthopaedic practice. *J Am Acad Orthop Surg* 7: 291–299, 1999.
12. **Carlsson SI, Bertilaccio MT, Ballabio E, Maier JA.** Endothelial stress by gravitational unloading: effects on cell growth and cytoskeletal organization. *Biochim Biophys Acta* 1642: 173–179, 2003.
13. **Clark RAF.** Wound repair. In: *The Molecular and Cellular Biology of Wound Repair*, edited by Clark RAF. New York: Plenum, 1995, p. 3–21.
14. **Cogoli A.** Signal transduction in T lymphocytes in microgravity. *Gravit Space Biol Bull* 10: 5–16, 1997.
15. **Cogoli A, Cogoli-Greuter M.** Activation and proliferation of lymphocytes and other mammalian cells in microgravity. *Adv Space Biol Med* 6: 33–79, 1997.
16. **Colleran PN, Wilkerson MK, Bloomfield SA, Suva LJ, Turner RT, Delp MD.** Alterations in skeletal perfusion with simulated microgravity: a possible mechanism for bone remodeling. *J Appl Physiol* 89: 1046–1054, 2000.
17. **Convertino VA, Bloomfield SA, Greenleaf JE.** An overview of the issues: physiological effects of bed rest and restricted physical activity. *Med Sci Sports Exerc* 29: 187–190, 1997.
18. **Crandall CG, Shibasaki M, Wilson TE, Cui J, Levine BD.** Prolonged head-down tilt exposure reduces maximal cutaneous vasodilator and sweating capacity in humans. *J Appl Physiol* 94: 2330–2336, 2003.
19. **Davidson JM, Aquino AM, Woodward SC, Wilfinger WW.** Sustained microgravity reduces intrinsic wound healing and growth factor responses in the rat. *FASEB J* 13: 325–329, 1999.
20. **DiPietro LA, Polverini PJ.** Angiogenic macrophages produce the angiogenic inhibitor thrombospondin 1. *Am J Pathol* 143: 678–684, 1993.
21. **DiPietro LA, Polverini PJ.** Role of the macrophage in the positive and negative regulation of wound neovascularization. *Behring Inst Mitt*: 238–247, 1993.
22. **DiPietro LA, Nissen NN.** Angiogenic mediators in wound healing. In: *Angiogenesis: Models, Modulators, and Clinical Applications*, edited by Maragoudakis ME. New York: Plenum, 1998, p. 121–129.
23. **Dovi JV, He LK, DiPietro LA.** Accelerated wound closure in neutrophil-depleted mice. *J Leukoc Biol* 73: 448–455, 2003.
24. **Emery CF, Kiecolt-Glaser JK, Glaser R, Malarkey WB, Frid DJ.** Exercise accelerates wound healing among healthy older adults: a preliminary investigation. *J Gerontol A Biol Sci Med Sci* 60: 1432–1436, 2005.
25. **Fortney SS, VS, Greenleaf JE.** The physiology of bed rest. In: *Handbook of Physiology. Environmental Physiology.* Bethesda, MD: Am. Physiol. Soc, 1996, sect. 4, vol. II, chapt. 39, p. 889–939.
26. **Fukushima T, Nakayama K, Monma M, Sekizawa K, Sasaki H.** Depression of T helper-1 and tuberculin responses in older bed-bound patients. *J Am Geriatr Soc* 47: 259–260, 1999.
27. **Gallagher SM.** Morbid obesity: a chronic disease with an impact on wounds and related problems. *Ostomy Wound Manage* 43: 18–24, 26–17, 1997.
28. **Gashev AA, Delp MD, Zawieja DC.** Inhibition of active lymph pump by simulated microgravity in rats. *Am J Physiol Heart Circ Physiol* 290: H2295–H2308, 2006.
29. **Gille H, Kowalski J, Li B, LeCouter J, Moffat B, Zioncheck TF, Pelletier N, Ferrara N.** Analysis of biological effects and signaling properties of Flt-1 (VEGFR-1) and KDR (VEGFR-2). A reassessment using novel receptor-specific vascular endothelial growth factor mutants. *J Biol Chem* 276: 3222–3230, 2001.
30. **Ilyina-Kakueva EI, Burkovskaya TE.** The microgravity effect on a repair process in *M. soleus* of the rats flown on Cosmos-2044. *Physiologist* 34: S141–S142, 1991.
31. **Jung CK, Chung S, Lee YY, Hwang SH, Kang CS, Lee KY.** Monocyte adhesion to endothelial cells increases with hind-limb unloading in rats. *Aviat Space Environ Med* 76: 720–725, 2005.
32. **Krasnoff J, Painter P.** The physiological consequences of bed rest and inactivity. *Adv Ren Replace Ther* 6: 124–132, 1999.
33. **Li Z, Rivera CA, Burns AR, Smith CW.** Hindlimb unloading depresses corneal epithelial wound healing in mice. *J Appl Physiol* 97: 641–647, 2004.
34. **Marqueste T, Alliez JR, Alluin O, Jammes Y, Decherchi P.** Neuromuscular rehabilitation by treadmill running or electrical stimulation after peripheral nerve injury and repair. *J Appl Physiol* 96: 1988–1995, 2004.
35. **Martel E, Champeroux P, Lacolley P, Richard S, Safar M, Cuche JL.** Central hypervolemia in the conscious rat: a model of cardiovascular deconditioning. *J Appl Physiol* 80: 1390–1396, 1996.

36. **Martinez DA, Vailas AC, Vanderby R Jr, Grindeland RE.** Temporal extracellular matrix adaptations in ligament during wound healing and hindlimb unloading. *Am J Physiol Regul Integr Comp Physiol* 293: R1552–R1560, 2007.
37. **Molina PE, McClain C, Valla D, Guidot D, Diehl AM, Lang CH, Neuman M.** Molecular pathology and clinical aspects of alcohol-induced tissue injury. *Alcohol Clin Exp Res* 26: 120–128, 2002.
38. **Moos PJ, Fattaey HK, Johnson TC.** Cell proliferation inhibition in reduced gravity. *Exp Cell Res* 213: 458–462, 1994.
39. **Morey-Holton ER, Globus RK.** Hindlimb unloading rodent model: technical aspects. *J Appl Physiol* 92: 1367–1377, 2002.
40. **Mousley M.** Diabetes and its effect on wound healing and patient care. *Nurs Times* 99: 70, 73–74, 2003.
41. **Murdaca G, Setti M, Brenci S, Fenoglio D, Lantieri P, Indiveri F, Puppo F.** Modifications of immunological and neuro-endocrine parameters induced by antiorthostatic bed-rest in human healthy volunteers. *Minerva Med* 94: 363–378, 2003.
42. **Nerem RM.** Shear force and its effect on cell structure and function. *ASGSB Bull* 4: 87–94, 1991.
43. **Norris SO, Provo B, Stotts NA.** Physiology of wound healing and risk factors that impede the healing process. *AACN Clin Issues Crit Care Nurs* 1: 545–552, 1990.
44. **Provenzano PP, Martinez DA, Grindeland RE, Dwyer KW, Turner J, Vailas AC, Vanderby R Jr.** Hindlimb unloading alters ligament healing. *J Appl Physiol* 94: 314–324, 2003.
45. **Radek KA, Matthies AM, Burns AL, Heinrich SA, Kovacs EJ, Dipietro LA.** Acute ethanol exposure impairs angiogenesis and the proliferative phase of wound healing. *Am J Physiol Heart Circ Physiol* 289: H1084–H1090, 2005.
46. **Ray CA, Vasques M, Miller TA, Wilkerson MK, Delp MD.** Effect of short-term microgravity and long-term hindlimb unloading on rat cardiac mass and function. *J Appl Physiol* 91: 1207–1213, 2001.
47. **Reed M, Teruhiko K, Puolakkainen P.** Wound repair in aging: a review. In: *Methods in Molecular Medicine*, edited by DiPietro L, Burns AL. Totowa: Humana, 2003, p. 217–240.
48. **Rico RM, Ripamonti R, Burns AL, Gamelli RL, DiPietro LA.** The effect of sepsis on wound healing. *J Surg Res* 102: 193–197, 2002.
49. **Riley DA, Ilyina-Kakueva EI, Ellis S, Bain JL, Slocum GR, Sedlak FR.** Skeletal muscle fiber, nerve, and blood vessel breakdown in space-flown rats. *FASEB J* 4: 84–91, 1990.
50. **Russell L.** The importance of patients' nutritional status in wound healing. *Br J Nurs* 10: S42, S44–49, 2001.
51. **Schmitt DA, Schwarzenberg M, Tkaczuk J, Hebrard S, Brandenberger G, Mauco G, Cogoli-Greuter M, Abbal M.** Head-down tilt bed rest and immune responses. *Pflügers Arch* 441: R79–R84, 2000.
52. **Singer AJ, Clark RA.** Cutaneous wound healing. *N Engl J Med* 341: 738–746, 1999.
53. **Swift ME, Kleinman HK, DiPietro LA.** Impaired wound repair and delayed angiogenesis in aged mice. *Lab Invest* 79: 1479–1487, 1999.
54. **Tang DG, Conti CJ.** Endothelial cell development, vasculogenesis, angiogenesis, and tumor neovascularization: an update. *Semin Thromb Hemost* 30: 109–117, 2004.
55. **Thomas DR.** Age-related changes in wound healing. *Drugs Aging* 18: 607–620, 2001.
56. **Vanderby R Jr, Vailas AC, Graf BK, Thielke RJ, Ulm MJ, Kohles SS, Kunz DN.** Acute modification of biomechanical properties of the bone-ligament insertion to rat limb unweighting. *FASEB J* 4: 2499–2505, 1990.
57. **Watters C, Tredget E.** Nutrition and wound healing. *Canadian J CME*: 65–74, 2002.
58. **Wilkerson MK, Lesniewski LA, Golding EM, Bryan RM Jr, Amin A, Wilson E, Delp MD.** Simulated microgravity enhances cerebral artery vasoconstriction and vascular resistance through endothelial nitric oxide mechanism. *Am J Physiol Heart Circ Physiol* 288: H1652–H1661, 2005.
59. **Woessner J.** The determination of hydroxyproline in tissue and protein samples containing small proportions of the imino acid. *Arch Biochem Biophys* 93: 440–447, 1961.
60. **Yamaguchi Y, Yoshikawa K.** Cutaneous wound healing: an update. *J Dermatol* 28: 521–534, 2001.